

Role of Anthranilic Acid in Background Levels of Sulfonamide in Porcine Livers when Determined by the Tishler Method

OWEN W. PARKS

Agricultural Research Service, Eastern Regional Research Center, Philadelphia, PA 19118

Anthranilic acid occurs in excised swine livers as a result of temperature abuse before and/or after freezing. The tryptophan metabolite is the major source of the variable non-sulfonamide background level frequently encountered in the Tishler method for determining sulfonamide residues in swine livers. Diazotized anthranilic acid reacts slowly with *N*-1-(naphthyl)ethylenediamine [$k(s^{-1}) = 5.79 (\pm 0.07) \times 10^{-5}$] and the final background level depends on the time elapsed between addition of the coupling agent and spectrophotometric determination. Kynurenine was tentatively identified as a minor source of the non-sulfonamide background level.

The procedure of Tishler et al. (1) (commonly referred to as the Tishler method), specifying the Bratton-Marshall (2) reaction, is the unofficial method of choice used by the regulatory agencies for determining sulfonamide residues in edible animal tissues. The colorimetric procedure, based on the coupling of *N*-1-(naphthyl)ethylenediamine to diazotized primary aromatic amines, is not specific for sulfonamides; therefore, the procedure is potentially subject to erroneous results. It is not unusual to conclude, on the basis of results of the Tishler method, apparent sulfonamide residues of 0.01–0.05 ppm in liver tissues obtained from animals maintained in carefully controlled sulfonamide-free environments (3). Furthermore, it is well known that higher values can occur if the tissues are subjected to temperature abuse before analysis (3). Temperature abuse may be responsible, at least partially, for wide variations in results on the same samples assayed by different laboratories (4).

In a recent publication (5), we identified anthranilic acid (*o*-aminobenzoic acid), a naturally occurring Bratton-Marshall-positive compound, in swine liver extracts analyzed by a slight modification of the Tishler method. The present manuscript defines the role of anthranilic acid in the variable "background level" of the Bratton-Marshall Tishler method for sulfonamide

residues in swine livers and presents other observations made during the course of the study.

Experimental

Reagents

(a) *Solvents*.—Acetone, hexane (Mallinckrodt, Inc., St. Louis, MO), and ethyl acetate (Burdick and Jackson Laboratories, Inc., Muskegon, MI 49442) were distilled in glass; chloroform, Baker Analyzed reagent (J. T. Baker Chemical Co., Phillipsburg, NJ 08665).

(b) *Chemicals*.—Anthranilic acid (Eastman Kodak, Rochester, NY 14650); kynurenine (Sigma Chemical Co., St. Louis, MO 63178); *o*-aminoacetophenone (Aldrich Chemical Co., Milwaukee, WI 53233).

(c) *N*-1-(Naphthyl)ethylenediamine (NEDA) dihydrochloride.—0.1% aqueous solution (Sigma Chemical Co.).

(d) *Sodium nitrite*.—0.1% aqueous solution.

(e) *Ammonium sulfamate*.—0.5% aqueous solution.

Apparatus

(a) *Tissue grinder*.—Brinkmann Polytron® homogenizer (Brinkmann Instruments Inc., Westbury, NY 11590).

(b) *Centrifuge*.—Servall Superspeed centrifuge, type SS-1 Rotor (Ivan Sorvall, Inc., Norwalk, CT).

(c) *Vortex stirrer*.—Super Mixer (Lab-Line Instruments, Inc., Melrose Park, IL 60160).

(d) *Spectrophotometer*.—Hitachi-Perkin Elmer Model 139 UV-visible spectrophotometer operated at 545 nm.

(e) *Thin layer chromatographic apparatus and reagents*.—2.5 × 10 cm precoated (250 μm) silica gel G glass plates (Analtech, Inc., Newark, DE 19711). Anthranilic acid solvent system: ethyl acetate-methanol (4 + 1); *o*-aminoacetophenone solvent system: hexane-ethyl acetate (4 + 1).

(f) *UV source*.—Chromato-Vue (Ultra-Violet Products, Inc., San Gabriel, CA 91778).

Liver Samples

Swine livers were obtained from a local slaughter house immediately after slaughter. After being excised from the animals, 4 adjacent portions (25–30 g) were sliced from a section of the organ. One portion (zero abuse) was frozen immediately in crushed dry ice and the others were held 1, 3, and 5 h, respectively, at ambient temperature before freezing at -15°C .

Modified Bratton-Marshall Tishler Method (BMTM)

Fifteen mL chloroform-acetone (1 + 1) was added to 5 g partially thawed tissue in a 50 mL polypropylene centrifuge tube and the mixture was homogenized at low speed for 1 min. The homogenate was centrifuged 2 min at 3500 rpm. The solvent was removed with a disposable Pasteur pipet and filtered through glass wool packed in a super Pasteur pipet. The filtrate was collected in a 50 mL polypropylene screw-cap centrifuge tube. The tissue was re-homogenized with additional 15 mL chloroform-acetone (1 + 1) and centrifuged, and the solvent was recovered and filtered through glass wool into the 50 mL screw-cap centrifuge tube. The combined extracts were evaporated just to dryness under a stream of nitrogen at 50°C . The residue was dissolved in 0.6 mL acetone and 15 mL hexane with vortex mixing. The solvent was extracted with two 1.25 mL portions of 1N HCl in a rocking motion for 3 min followed by centrifugation at 3500 rpm for 2 min. The aqueous layers were recovered with a disposable Pasteur pipet and filtered through glass wool packed in a Pasteur pipet into a 10 mL graduated cylinder. The entire procedure was repeated on a second 5 g sample, and the HCl extracts from each sample were combined, diluted to 6 mL with 1N HCl, mixed thoroughly, and divided into two 3 mL portions in 9 mL screw-cap specimen vials. To each sample and a 3 mL blank sample of 1N HCl, 0.2 mL 0.1% aqueous sodium nitrite was added. The solutions were mixed thoroughly, allowed to stand for 3 min, 0.2 mL 0.5% aqueous ammonium sulfamate was added, and solutions mixed thoroughly and allowed to stand for 2 min. To one sample and the HCl blank, 0.2 mL 0.1% aqueous NEDA was added; to the remaining sample (control), 0.2 mL water was added. Solutions were mixed thoroughly and allowed to stand in the dark. Absorbances were determined at 545 nm in a 1 cm cell at 0.25, 1.0, 2.0, and 4.0 h after addition of NEDA.

Anthranilic Acid – BM Reaction

To determine reaction rate, 2.64 μg anthranilic acid/3 mL 1N HCl was subjected to the BM reaction according to the procedure outlined above. Absorbances were obtained at 545 nm (maximum absorption, 550 nm) in 1 cm cell at various intervals following addition of NEDA.

Absorptivities at 1, 2, and 4 h reaction times were obtained on standard solutions of 0.22–3.08 μg anthranilic acid/3 mL 1N HCl.

Results and Discussion

Preliminary studies on numerous swine livers, using the previously described (6) thin layer chromatographic (TLC) screening procedure for sulfamethazine and sulfathiazole, demonstrated that the majority of samples frozen in dry ice immediately after being excised from the animal contained only trace amounts of anthranilic acid. However, if the livers were abused, that is, held at ambient temperatures before freezing, the concentration of the metabolite increased significantly in about half of the livers analyzed, and the concentration increased with increased time of abuse. The same results were observed by TLC of methanol effluents from Chromosorb 102 which was shown previously (5) to adsorb anthranilic acid from liver extracts obtained by the Tishler method. Based on these initial observations, the actual contribution of anthranilic acid to the variable background levels observed in the Tishler method for determining sulfonamide residues in swine livers was investigated.

Studies on anthranilic acid in the Bratton-Marshall reaction revealed that, in contrast to diazotized sulfonamide which reacts in 15 min, diazotized anthranilic acid reacted more slowly with the coupling agent (NEDA). The reaction for a series of known concentrations of anthranilic acid was complete, for practical purposes, within 24 h, with a mean absorptivity of 230 (L/g/cm light path). Hence by using the absorptivity equation ($E = A/(c \times l)$, where E = absorptivity; A = absorbance; c = concentration in g/L; and l = length of light path in cm), it is possible to determine the amount of diazotized anthranilic acid in a standard solution reacted at time t . An analysis of results obtained on a known concentration of anthranilic acid showed the reaction to be first-order and could be expressed mathematically by $kt = \ln a^{\circ}/a$ (k = rate constant, a° = initial concentration, and a = concentration remaining at time t). Using this equation, the slope of a straight line, determined by the method of least squares, corresponding to

the reaction rate [$k(s^{-1}) = 5.79 (\pm 0.07) \times 10^{-5}$] was obtained (0.07 = mean deviation of duplicate samples) ($r = 0.9997$). The reaction was calculated to be 95% complete in 14.4 h. Spectrophotometric studies on liver extracts spiked with anthranilic acid demonstrated, however, that holding samples for extended periods before spectrophotometric determination, in addition to being impractical, resulted in the development of a non-Bratton-Marshall blue color and an occasional cloudy reaction mixture. To overcome these limitations and still obtain quantitative anthranilic acid data, mean absorptivity values of known concentrations of anthranilic acid in the reaction were determined at 1 ($E = 46$), 2 ($E = 83$), and 4 h ($E = 136$) reaction times.

Table 1 compares the results (in absorbance units) of 2 samples of swine livers held at ambient temperature for 0, 1, 3, and 5 h before freezing and analyzed by the modified Tishler method. Absorbances were determined after 0.25, 1.0, 2.0, and 4.0 h following addition of NEDA. In most samples analyzed, trace amounts of sulfamethazine were observed by the TLC screening procedure which may account for all or a portion of the absorbance of non-abused samples read after 0.25 h color development. As shown by both TLC and spectrophotometric determination, liver A developed very little anthranilic acid, even in the sample abused for 5 h. In contrast, liver B developed significant amounts, the concentration of which increased with increasing time of abuse. As important to the final results is the increase in absorbance as the time differential between addition of NEDA and spectrophotometric determination increased. For example, the 5 h abused liver B read 0.25 h after addition of NEDA had an absorbance of 0.009, equivalent to 0.034 ppm sulfamethazine (0.1 ppm sulfamethazine, the regulatory violative level, in 5 g liver has an absorbance of 0.026). However, if the reaction was allowed to proceed for 1 h, the maximum time recommended (3), 0.077 ppm apparent sulfonamide (based on sulfamethazine) would be concluded based on the absorbance of 0.020, but actually a maximum of 0.011 ppm (based on non-abuse, 0.25 h absorbance) is present in the sample.

Applying the absorptivity values determined at 1, 2, and 4 h reaction time to the absorbances obtained on liver B (minus non-abuse, 0.25 h absorbance) suggested that small amounts of other non-sulfonamide Bratton-Marshall-positive compounds were also present in the 3 and 5 h abused samples. TLC on extracts obtained by the screening procedure from abused livers with

Table 1. Effect (measured in absorbance unit) of abuse and elapsed time before spectrophotometric determination on results for sulfonamide residues in swine livers

Abuse time, h ^a	Absorbance time, h ^b			
	0.25	1.0	2.0	4.0
Liver Sample A				
0	0.002	0.002	0.002	0.002
1	0.002	0.004	0.005	0.007
3	0.004	0.007	0.009	0.011
5	0.004	0.007	0.009	0.011
Liver Sample B				
0	0.003	0.004	0.006	0.009
1	0.003	0.006	0.010	0.013
3	0.005	0.011	0.016	0.021
5	0.009	0.020	0.032	0.049

^a Time fresh swine liver held at ambient temperature before freezing.

^b Hours following addition of NEDA reagent.

significant amounts of anthranilic acid revealed trace amounts of an unknown positive compound at the origin following solvent development. The unknown was tentatively identified as kynurenine on the basis of the odor, fluorescence, positive Bratton-Marshall reaction, and TLC characteristics of its alkaline hydrolysis product *o*-aminoacetophenone (7). Kynurenine is not considered a major factor in the Tishler method because of its low solubility in organic solvents and a molar absorptivity of 8500 in the Bratton-Marshall reaction (ϵ sulfamethazine = 52 500). It is estimated that kynurenine contributed less than 0.01 ppm to 5 h abused liver B sample reported in Table 1.

To determine the effect of frozen storage on the development of anthranilic acid in swine liver, fresh samples were obtained and each sample was divided into 2 portions. One portion of each sample was abused before freezing. The second portion of each sample was frozen immediately and held at -15°C for 2-4 months before being thawed and abused. Typical results of these studies are presented in Table 2. Anthranilic acid did not develop during frozen storage. However, temperature abuse after freezing resulted in higher concentrations of anthranilic acid compared with abuse before freezing. This suggests that freezing and thawing significantly disrupts liver cells, thereby increasing the substrate (i.e., tryptophan or its metabolites) available for enzymatic degradation to anthranilic acid. The variability in individual

Table 2. Effect of swine liver abuse on formation of anthranilic acid (ppm)^a

Liver sample	Abuse treatment	Abuse time, h ^b			
		0	1	3	5
C	before freezing	0.03	0.03	0.05	0.04
	after freezing	0.03	0.04	0.08	0.09
D	before freezing	0.02	0.04	0.12	0.19
	after freezing	0.03	0.05	0.16	0.28

^a Based on 4 h absorbances (minus zero h abuse, 0.25 h reading).

^b Time swine liver was held at ambient temperature.

Table 3. Effects on abuse treatments before and after freezing on formation of anthranilic acid in swine liver

Abuse time, h ^a			Anthranilic acid, ppm ^b
Before freezing	After freezing	Total	
0	0	0	0.04
1	0	1	0.05
3	0	3	0.22
5	0	5	0.30
0	0	0	0.05
0	1	1	0.13
1	2	3	0.28
3	2	5	0.50

^a Time swine liver was held at ambient temperature.

^b Based on 4 h absorbances (minus zero h abuse, 0.25 h reading).

liver samples to develop significant amounts of anthranilic acid is again evident.

Table 3 demonstrates the additive effect of abusing samples before freezing and following frozen storage. Liver samples which had been previously abused 0, 1, and 3 h at ambient temperature before freezing were thawed after 1 month storage at -15°C and abused at ambient temperature for an additional 1, 2, and 2 h, respectively. As might be expected, anthranilic acid concentration increased in the doubly abused samples relative to samples abused only before freezing for the corresponding length of time.

The results of these studies establish anthranilic acid as the major source of the variable non-sulfonamide Bratton-Marshall background

levels encountered in the Tishler method for sulfonamide residues in swine livers. The variability in levels depends on the availability of precursor and/or the ability of individual livers to develop anthranilic acid as a result of temperature abuse before and/or after freezing, the length of temperature abuse, and the time between addition of NEDA to diazotized samples and spectrophotometric determination. Hence, it is erroneous to arbitrarily subtract a fixed background level from unknown liver samples as has been done in the past (3, 4) by regulatory agencies and research institutions. The need for a quantitative procedure free of background interferences is evident. Until such procedures are developed, increased care must be taken in sample handling, and the time of spectrophotometric determination must be standardized to 0.25 h following addition of NEDA to ensure that accurate and reproducible results are obtained by the Tishler method and similar Bratton-Marshall procedures.

REFERENCES

- (1) Tishler, F., Sutter, J. L., Bathish, J. N., & Hagman, H. E. (1968) *J. Agric. Food Chem.* **16**, 50-53
- (2) Bratton, A. C., & Marshall, E. K. (1939) *J. Biol. Chem.* **128**, 537
- (3) Horwitz, W. (1981) *J. Assoc. Off. Anal. Chem.* **64**, 104-130
- (4) Lloyd, W. E., Jenny, A. L., Cox, D. F., & Rottinghaus, G. E. (1981) *Am. J. Vet. Res.* **42**, 339-343
- (5) Parks, O. W., & Kimoto, W. I. (1982) *J. Assoc. Off. Anal. Chem.* **65**, 1285-1287
- (6) Parks, O. W. (1982) *J. Assoc. Off. Anal. Chem.* **65**, 632-634
- (7) Spacek, M. (1954) *Can. J. Biochem. Physiol.* **32**, 604